RHODORA, Vol. 105, No. 921, pp. 76–90, 2003

MOLECULAR SYSTEMATICS OF THE EASTERN NORTH AMERICAN *SILENE* (CARYOPHYLLACEAE): EVIDENCE FROM NUCLEAR ITS AND CHLOROPLAST *trn*L INTRON SEQUENCES

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ABSTRACT. This study examines the phylogenetic relationships of the nine *Silene* species endemic to eastern North America using nuclear ITS and chloroplast *trn*L intron sequence data. The ITS region is highly variable among taxa and is more phylogenetically informative than the less variable *trn*L intron DNA data. The ITS sequences indicate that the eastern North American taxa are not monophyletic, but instead occur in two clades that are nested within a clade including western species. *Silene* section *Occidentales* is also not monophyletic. The diverse floral morphologies of the eastern North American *Silene* appear to be evolutionarily labile. Neither floral morphology nor pollinator syndromes are conserved within clades. Both ITS and *trn*L intron data indicate that the hummingbird-pollinated taxa are not monophyletic. There is weak evidence for differences in the nuclear and chloroplast phylogenies that may be a result of reticulate evolution.

Key Words: Caryophyllaceae, ITS, *trn*L intron, *Silene*

With over 700 taxa, *Silene* is the largest genus within the Caryophyllaceae (Bittrich 1993; Greuter 1995). Though diversity is greatest in the Mediterranean region (Greuter 1995), approximately 50 taxa are endemic to North America, and nine taxa are found exclusively east of the Rocky Mountains (Hitchcock and Maguire 1947). The nine taxa endemic to eastern North America are presumably closely related, yet they are morphologically diverse (Hitchcock and Maguire 1947). The flowers of the nine taxa exhibit a large range of shapes, sizes, and colors (Table 1). Four of these taxa are among the few hummingbird-pollinated species endemic to eastern North America (Austin 1975). Though the characteristics of the eastern North American *Silene* are well described, their evolutionary relationships are unclear. This study uses DNA sequence analysis to examine the relationships within

Table 1. *Silene* taxa used in this study using the sectional classification of Chowdhuri (1957).

Species	Section	Floral Color
Eastern North American taxa		
S. caroliniana Walter		
subsp. *caroliniana* Walter	*Occidentales*	pink
subsp. *pensylvanica* R. T. Clausen	*Occidentales*	pink
subsp. *wherryi* (Small) R. T. Clausen	*Occidentales*	pink
S. nivea (Nutt.) Muhl. *ex* DC.	*Siphonomorpha*	white
S. ovata Pursh	*Paniculatae*	white
S. polypetala (Walter) Fernald & B. G. Schub.	*Occidentales*	pink
S. regia Sims	*Occidentales*	red
S. rotundifolia Nutt.	*Occidentales*	red
S. stellata Aiton	*Siphonomorpha*	white
S. subciliata B. L. Rob.	*Occidentales*	red
S. virginica L.	*Occidentales*	red
Western North American taxa		
S. californica Durand	*Occidentales*	red
S. lemmonii S. Watson	*Siphonomorpha*	white
S. petersonii Maguire	*Occidentales*	pink
Other taxa		
S. acaulis (L.) Jacq.		
subsp. *subacaulescens* (F. N. Williams) Hulten	*Nanosilene*	pink
S. furcata Raf.	*Physolychnis*	white
S. viscosa Pers.	*Chloranthae*	white

eastern North American *Silene* and surveys the evolution of floral diversity in these taxa.

Studies of *Silene* taxonomy and systematics are complicated by polyploidy and interspecific hybridization. The initial classifications of *Silene* focused on European and Asian taxa. Hitchcock and Maguire's (1947) study was the first thorough description and classification of the North American *Silene*. They applied Williams' (1896) subgeneric classification to the North American species, grouping the eastern North American *S. stellata* and *S. ovata* in subgenus *Eusilene* and the other seven taxa in subgenus *Melandryum*. Chowdhuri's (1957) taxonomic description of the entire genus divided *Silene* into 44 sections. He placed the white-flowered eastern North American taxon *S. ovata* alone among

North American taxa in section *Paniculatae,* subsection *Laciniatae.* The red-flowered, hummingbird-pollinated *S. regia, S. rotundifolia, S. subciliata,* and *S. virginica* were grouped in section *Occidentales,* and the white-flowered *S. nivea* and *S. stellata* were grouped in section *Siphonomorpha* (Table 1). Though not classified by Chowdhuri (1957), the pink-flowered *S. caroliniana* and *S. polypetala* fit in section *Occidentales* based on their ciliate claws and pilose filaments (pers. obs.).

Cytological studies and crossing experiments indicate substantial genetic separation between North American and European *Silene.* Kruckeberg (1960) observed that most North American taxa, including all seven eastern North American species he examined, have $2n = 48$ chromosomes, while many Eurasian species have $2n = 24$ chromosomes. Heaslip's (1950, 1951) crossing experiments found that crosses between the four North American and the European *S. latifolia* (Mill.) Britton & Rendle (*S. alba* Mill.) taxa produced no seed, but crosses between four North American taxa produced seed and sterile offspring. Kruckeberg's crosses between North American and European taxa also produced no progeny, and his extensive crosses among western (1961) and eastern (1963) North American *Silene* yielded mostly sterile hybrids. In one exception, crosses between the eastern North American taxa *S. virginica* and *S. caroliniana* produced fertile hybrids (Kruckeberg 1963). These hybrids are also noted to occur naturally (Mitchell and Uttal 1969; Steyermark 1963).

Recent molecular studies have examined *Silene* systematics at the generic (Desfeux and Lejeune 1996; Oxelman et al. 1997; Oxelman and Lidén 1995), sectional (Oxelman 1996), and species (Vellekoop et al. 1996) levels. However, these studies include few, if any, North American taxa. Oxelman and Lidén (1995) found that, based on nrDNA sequences, the eastern North American *S. rotundifolia* and the western North American *S. petersonii* from section *Occidentales* form a well supported clade among the sections *Physolychnis* and *Odontopetalae.*

This study focuses on the relationships within the eastern North American *Silene* using both nuclear and chloroplast DNA sequence data. Since previously described chromosome number, crossing, and molecular data suggest that many endemic North American *Silene* may be monophyletic, and the nine eastern North American taxa are geographically separated from the majority of North American taxa, the study examines if the eastern

North American taxa are monophyletic. Next, it addresses the monophyly of the sectional classifications, hummingbird pollination, and floral color of the eastern North American taxa. Finally, it will compare chloroplast and nuclear phylogenies for evidence of hybridization among taxa.

MATERIALS AND METHODS

Plant tissue was obtained from all nine of the *Silene* taxa endemic to eastern North America (east of the Rocky Mountains) including all three subspecies of *S. caroliniana* (Clausen 1939; Wilbur 1970; Table 2). Tissue from western North American taxa *S. californica,* section *Occidentales,* and *S. lemmonii,* section *Siphonomorpha,* was sampled from herbarium specimens. The trees were rooted with *S. acaulis,* a circumpolar taxon in section *Nanosilene* (Chowdhuri 1957). There are no endemic North American taxa in *Nanosilene* (Chowdhuri 1957), and previous molecular studies indicate that *S. acaulis* is a distant sister to the endemic North American taxa (Oxelman and Lidén 1995). Furthermore, ITS and *trn*L intron alignments are uncertain when including outgroups outside *Silene* (pers. obs.). For the ITS tree, we also included sequences from *S. viscosa* and *S. furcata,* European taxa that appear to be sister to the North American taxa (Oxelman and Lidén 1995), as outgroups. Six ITS sequences from GenBank, including all previously sequenced North American taxa and the two European sister taxa (Oxelman and Lidén 1995), were included in the phylogenetic analysis (Table 2).

DNA was extracted using the DNeasy plant mini kit (Qiagen Inc., La Jolla, CA). The *trn*L intron was amplified with universal primers c and d (Taberlet et al. 1991). The DNA was amplified for 35 cycles of the polymerase chain reaction (1 min. at 94°C, 30 sec. at 58°C, and 1 min. at 72°C) in 50 μl reactions containing 5 μl 10× *Taq* polymerase reaction buffer (Promega, Madison, WI), 0.2 mM dNTPs, 0.6 μM each primer, and 0.5 units *Taq* polymerase. ITS was amplified with primers Fred and Barney (Buckler and Holtsford 1996), located in the 18S and 26S nrDNA genes respectively. Various PCR protocols were followed running from 35 to 40 cycles (1 min. at 94°C, 30 sec. at 54–58°C, and 1 min. at 72°C) containing 5 μl Promega 10× *Taq* polymerase reaction buffer, 6.0 mM $MgCl_2$ total, 0.2 mM dNTPs, 10% DMSO, 0.4 μM each primer, and 0.5 units *Taq* polymerase.

Table 2. Accession table of *Silene* taxa used in this study. Key to sources: GB = Göteborg University herbarium, MO = Missouri Botanic Garden herabarium, NLU = Northeast Louisiana University herbarium, UMO = University of Missouri herbarium, GP = Green Plant Market Nursery, JA = Antonovics, JB = Burleigh, LG = Galloway, MP = Puterbaugh. The "-" in the GenBank numbers signifies that the accession number beings with "AY1164-".

Species	Source; Origin (GenBank #)	ITS/*trn*L Clone #
S. acaulis subacaulescens	MP; no voucher; Park Co., Colo. (AY116473 / -82)	1 / 1
S. acaulis	GB; *Ox. 2243*; garden (X86860)	12 / –
S. californica	UMO; *Morris 336*; Jackson Co., Ore. (AY116483)	– / 2
S. caroliniana caroliniana	JA; no voucher; Aiken Co., S. C. (AY116474 / -86)	2 / 3
S. caroliniana pensylvanica	JA; no voucher; Franklin Co., N. C. (AY116484)	– / 4
S. caroliniana wherryi	JA; no voucher; Jessamine Co., Ky. (AY116481 / -85)	3 / 5
S. furcata	GB; *Ox. 1887*; Sweden (X86859)	13 / –
S. lemmonii	UMO; *Kenney 391*; Siskiyou Co., Calif. (AY116487)	– / 6
S. nivea	UMO; *Rickett*; Tippecanoe Co., Ind. (AY116488)	– / 7
S. ovata	MO; *Kral 59396*; Marengo Co., Ala. (AY116475 / -89)	4 / 8
S. petersonii	GB; *Ox. 2239*; garden (X86886)	14 / –
S. polypetala	GP; no voucher; garden (AY116480)	5 / –
S. polypetala	MO; *Mohr 4000*; Ala. (AY116490)	– / 9
S. regia	JB; no voucher; Dade Co., Mo. (AY116476)	6 / –
S. regia	JB; no voucher; Dade Co., Mo. (AY116495)	– / 10
S. regia	GB; no voucher, Benton Co., Ark. (X86885)	16 / –
S. rotundifolia	JA; no voucher; Dickenson Co., Va. (AY116477 / -91)	7 / 11
S. rotundifolia	GB; *Ox. 2231*; garden (X86887)	15 / –
S. stellata	LG; no voucher; Giles Co., Va. (AY116477)	– / 12
S. stellata	JB; no voucher; Boone Co., Mo. (AY116472)	8 / –
S. subciliata	NLU; *79543*; Allen Par., La. (AY116471, -78 / -93)	9, 10 / 13
S. virginica	JB; no voucher; Wake Co., N. C. (AY116479 / -94)	11 / 14
S. viscosa	GB; *Ox. 2288*; Greece (X86831)	17 / –

All ITS and approximately half of the *trn*L intron PCR products were cut from a 1.5% low melt agarose gel and cleaned using a Geneclean III kit (Bio 101, La Jolla, CA). The remaining *trn*L intron PCR products were cleaned directly using the same kit. All clean PCR products were then ligated into a pGEM-T vector system (Promega, Madison, WI) and were cloned into DH5α competent cells using the protocol from the Promega pGEM-T vector system technical manual. Transformed cells were grown in LB medium for 18 to 24 hours at 37°C while shaking. The plasmid vectors were extracted from the cell culture using the Promega Wizard Plus miniprep kit (Promega, Madison, WI). Miniprep products were sequenced in both the forward and reverse direction at the University of Missouri DNA core facility using an ABI 377 automated sequencer.

Forward and reverse traces for each sequence were compared and edited in Seqman (DNASTAR, Madison, WI) to construct a consensus sequence. Both *trn*L intron and ITS sequences were aligned in Megalign (DNASTAR, Madison, WI), and the alignments were manually adjusted. The alignments are available on TreeBASE. A parsimony analysis of each dataset was performed using a branch and bound search retaining 100 parsimonious trees on PAUP* version 4.0b2 (Swofford 1999). Gaps were treated as a fifth base, and preliminary analysis determined that gap handling had no major effect on the overall topology of either ITS or the *trn*L intron trees. One hundred nonparametric bootstrap replicates of each dataset were generated using the same search options to test the support of the branches on each phylogeny. A Bayesian analysis was conducted on the ITS dataset using MrBayes version 2.01 (Huelsenbeck 2000). The Bayesian analysis used a general reversible model (REV; e.g., Yang 1994a) with rate variation among nucleotides following a discrete gamma distribution with four rate categories (Yang 1994b). Gaps were treated as missing data in the Bayesian analysis. The MCMC search consisted of four chains, three of which were heated to a temperature of 0.2 (Huelsenbeck 2000). The Markov chain was sampled once every 100 generations for 1,000,000 generations. The posterior probability was then calculated from a consensus tree of all trees sampled after the Markov chain reached stationarity (after 30,000 generations). The somewhat limited taxon overlap between the two datasets made a combined analysis problematic.

RESULTS

The ITS alignment contained 662 characters, 420 of which were constant and 119 of which were parsimony informative. The parsimony search found a single most parsimonious tree. The most parsimonious tree had a length of 354 steps, a consistency index of 0.825, and a retention index of 0.786 (Figure 1). There was little variation within taxa and no evidence of divergent ITS paralogs. The two sequences from *Silene acaulis, S. regia, S. rotundifolia,* and *S. subciliata* each formed clades with bootstrap support values of 99% or 100%. The two subspecies of *S. caroliniana* were in a strongly supported clade with *S. virginica* and *S. stellata* (Figure 1). *Silene caroliniana* subspecies are geographically separated, morphologically distinct (Clausen 1939), and naturally hybridize with *S. virginica* (Mitchell and Uttal 1969; Steyermark 1963) and *S. polypetala* (Georgia Department of Natural Resources, pers. comm). Therefore, the distance between the two *S. caroliniana* sequences may be the result of genetic divergence between the subspecies or hybridization.

The eastern North American *Silene* formed two distinct clades in the ITS phylogeny (Figure 1). The white-flowered *S. ovata* and the red-flowered, hummingbird-pollinated *S. regia* and *S. subciliata* formed one clade with bootstrap support of 79%. *Silene rotundifolia, S. polypetala, S. caroliniana, S. virginica,* and *S. stellata* composed the second clade with bootstrap support of 64%. Within this second clade there was a very strongly supported clade containing the white-flowered *S. stellata,* the pink-flowered *S. caroliniana,* and the red-flowered *S. virginica.* The western North American *S. petersonii* was basal to this second clade, though bootstrap support was lacking. The European *S. furcata* and *S. viscosa* and circumpolar *S. acaulis* were basal to all North American taxa (Figure 1).

The basal position of the western North American *S. petersonii* relative to one clade of eastern North American taxa indicates that the eastern North American taxa may not be monophyletic. A parsimony search using a constraint tree that enforces the monophyly of the eastern North American taxa found a most parsimonious tree with 357 steps. A nonparametric Templeton test (Templeton 1983) could not reject the null hypothesis that support for the most parsimonious tree is significantly better than the constraint tree (p-value = 0.083).

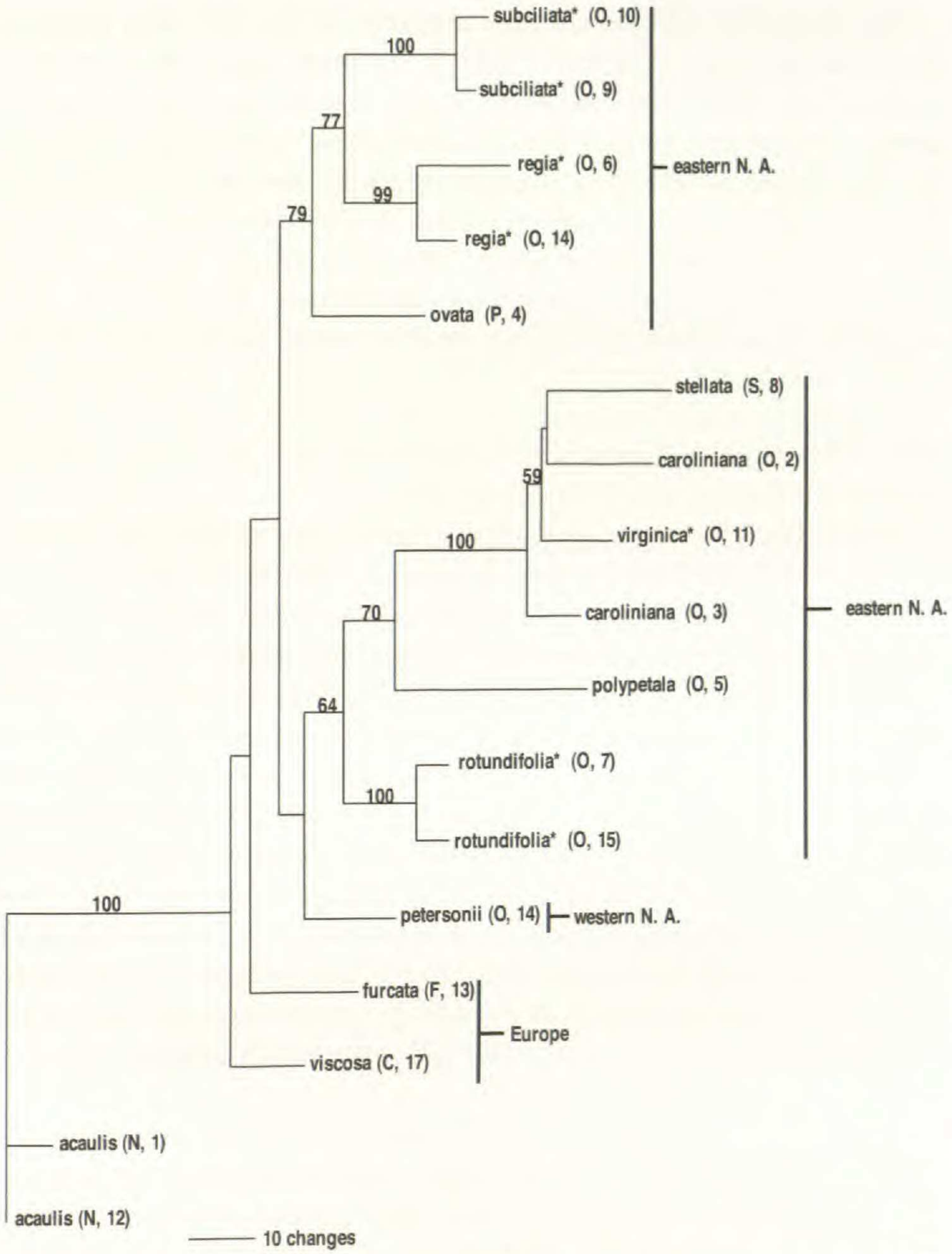

Figure 1. Phylogram of *Silene* taxa from a parsimony analysis of ITS sequences treating gaps as a fifth base. All bootstrap values above 50 are on the branches. The letters in parentheses represent the sectional classification of the taxa: C = *Chloranthae*, N = *Nanosilene*, O = *Occidentales*, P = *Paniculatae*, F = *Physolychnis*, S = *Siphonomorpha*. The numbers in parentheses are the sequence numbers from the accession table (Table 2). A star (*) by a taxon indicates hummingbird pollination.

The Bayesian and parsimony analysis of the ITS data support the same topology (Figures 1 and 2). In most cases, the posterior probabilities from the Bayesian analysis exceeded the nonparametric bootstrap values from the parsimony tree (Figures 1 and 2). The Bayesian analysis strongly supports the back branches of the phylogeny that are unsupported in the parsimony analysis. The Bayesian analysis supports the monophyly of the North American *Silene* with a posterior probability of 100%. It also supports the position of *S. petersonii* as sister to the clade of the eastern North American *S. stellata, S. virginica, S. caroliniana, S. polypetala,* and *S. rotundifolia* with a posterior probability of 95%. This further supports the hypothesis that the eastern North American *Silene* are not monophyletic.

There was little variation among *Silene* taxa in the *trn*L intron. Of the 676 characters in the alignment, 506 were constant and only 34 were parsimony informative. Therefore, the *trn*L intron did not resolve the relationships within the eastern North American *Silene* as well as ITS did (Figure 3). The branch and bound search retained two nearly identical most parsimonious trees, each with 191 total steps. The consistency index was 0.932 and the retention index was 0.787. The consensus tree supports the basal position of the western North American *S. californica* to the eastern North American taxa with a bootstrap value of 73%. The analysis also supports a clade of *S. carolinana, S. polypetala,* and *S. virginica* with bootstrap support of 71% (Figure 3). A clade with the white-flowered *S. nivea* and *S. stellata* and the red, hummingbird-pollinated *S. regia* has 62% bootstrap support (Figure 3).

The lack of resolution of the chloroplast *trn*L intron phylogeny makes it difficult to assess potential conflict between the nuclear and chloroplast phylogenies. Both phylogenies indicate a close relationship among *Silene caroliniana, S. polypetala, S. virginica,* and *S. stellata.* The position of *S. regia* is the most different in the ITS and *trn*L intron phylogenies (Figures 1, 2, and 3).

DISCUSSION

Though not conclusive, ITS evidence indicates that the eastern North American *Silene* are not monophyletic (Figures 1 and 2). The parsimony analysis lacks bootstrap support for the placement of the western North American *S. petersonii* in a clade among

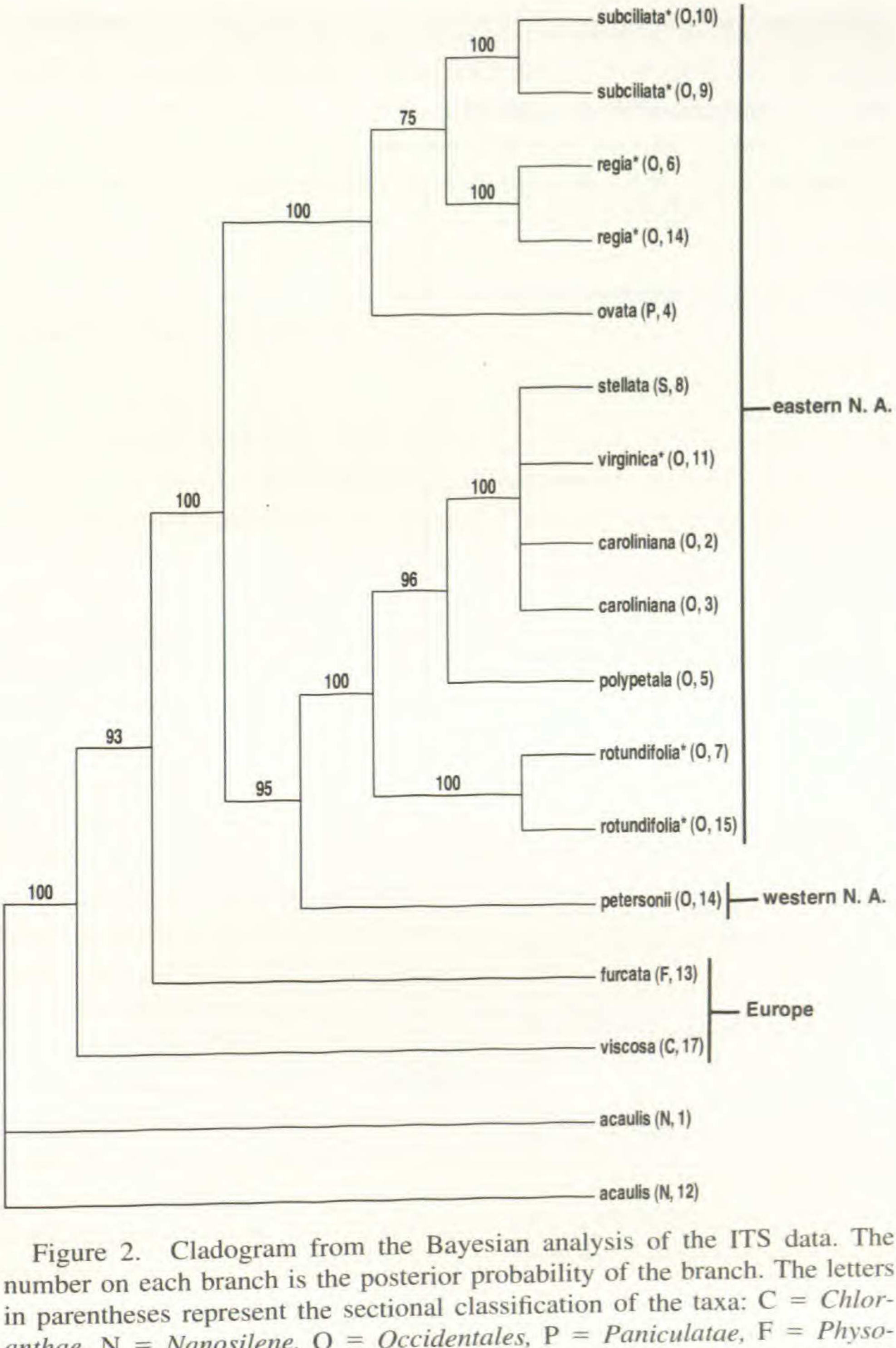

Figure 2. Cladogram from the Bayesian analysis of the ITS data. The number on each branch is the posterior probability of the branch. The letters in parentheses represent the sectional classification of the taxa: C = *Chloranthae*, N = *Nanosilene*, O = *Occidentales*, P = *Paniculatae*, F = *Physolychnis*, S = *Siphonomorpha*. The numbers in parentheses are the sequence numbers from the accession table (Table 2). A star (*) by a taxon indicates hummingbird pollination.

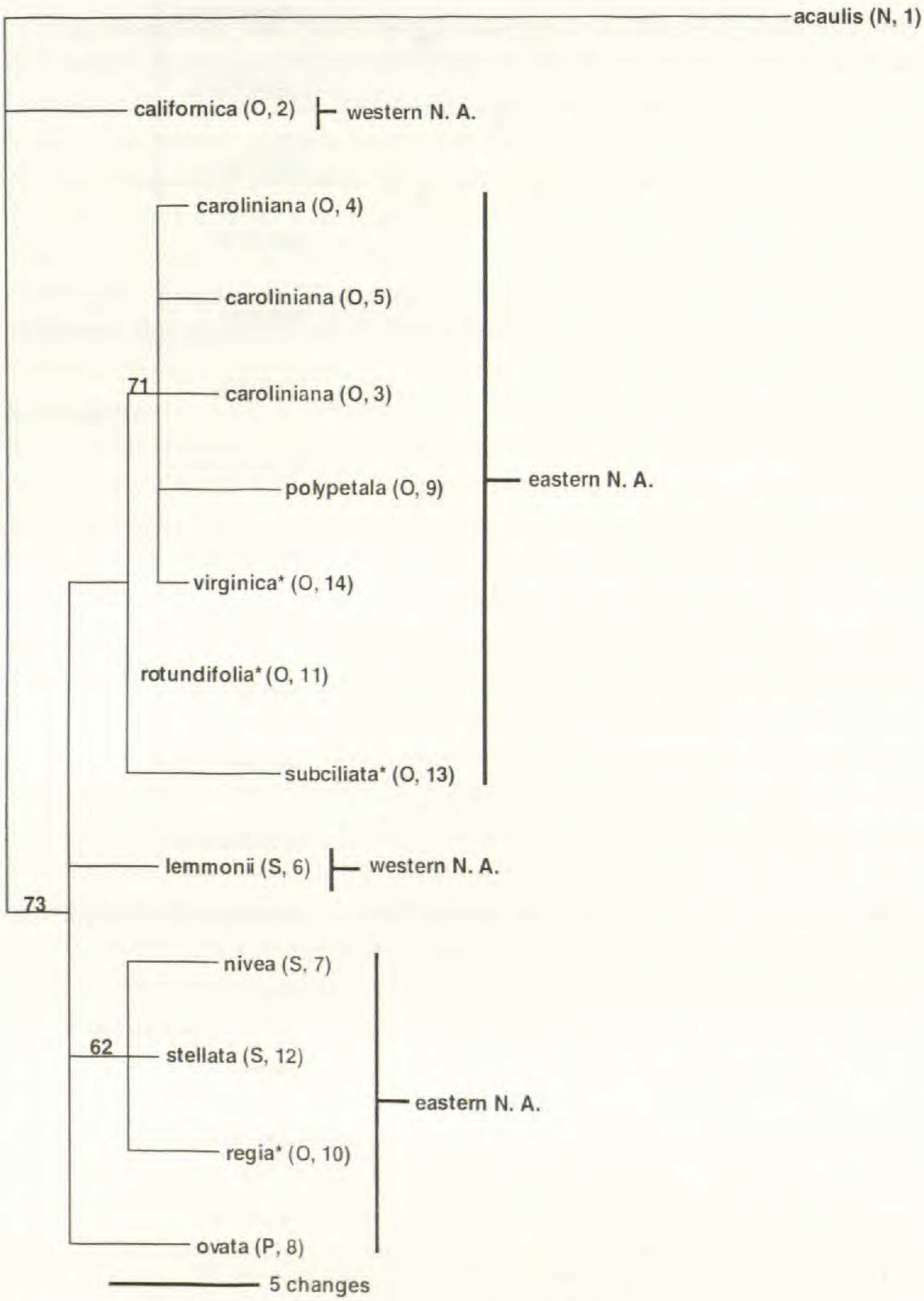

Figure 3. Phylogram of *Silene* taxa based on a consensus tree from parsimony analysis of the *trn*L intron. The letters in parentheses represent the sectional classification of the taxa: N = *Nanosilene*, O = *Occidentales*, P = *Paniculatae*, S = *Siphonomorpha*. Bootstrap values above 50 are shown. The numbers in parentheses are the sequence numbers from the accession table (Table 2). A star (*) by a taxon indicates hummingbird pollination.

eastern North American taxa (Figure 1). However, the Bayesian analysis strongly supports the position of *S. petersonii* as sister to one of two eastern North American clades (Figure 2). The Templeton test to reject the monophyly of the eastern North American taxa was nearly significant, but the short sequence length and lack of variation among North American *Silene* contributed to the failure to reject the null hypothesis. The *trn*L intron lacks the variation to test the monophyly of the eastern North American taxa (Figure 3).

The ITS phylogeny does provide evidence of the evolutionary lability of floral morphology and pollination systems within the eastern North American *Silene*. The clades are not organized with respect to floral morphology or pollinator syndrome. The red, hummingbird-pollinated *S. virginica* forms a strong clade with the pink-flowered *S. caroliniana* and the white-flowered *S. stellata* (Figures 1 and 2). The white-flowered taxon *S. ovata* forms a well-supported clade with the red, hummingbird-pollinated *S. regia* and *S. subciliata*. Neither red-, pink-, nor white-flowered taxa are monophyletic. Hitchcock and Maguire (1947) and Oxelman and Lidén (1995) previously noted the extensive homoplasy of floral traits within *Silene*. Different reproductive systems have also evolved multiple times within *Silene* (Desfeux et al. 1996). This study further emphasizes the great lability of floral traits in *Silene*, and demonstrates that homoplasies occur at a very low taxonomic level among closely related taxa in the same section. Chowdhuri's (1957) section *Occidentales* is also not monophyletic. Both *S. stellata*, section *Siphonomorpha*, and *S. ovata*, section *Paniculate*, are found within clades containing only *Occidentales* taxa (Figures 1 and 2).

The two eastern North American clades partially correspond to phenology and geography. *Silene polypetala*, *S. caroliniana*, and *S. virginica* all bloom in the spring in similar habitats in the southeastern United States. While *S. stellata* flowers later in the summer, its distribution overlaps with that of *S. caroliniana*, *S. virginica*, and *S. polypetala*. *Silene ovata*, *S. regia*, and *S. subciliata*, grouped together in this phylogeny, flower latest in the summer and fall. The closely related *S. regia* and *S. subciliata* are the only two eastern North American taxa with distributions almost totally west of the Appalachians. The geographic proximity of the genetically similar taxa may be the result of more recent speciation or gene flow among the taxa.

Though the *trn*L intron phylogeny contains few strongly supported clades, it also indicates the evolutionary lability of floral morphology. The hummingbird-pollinated *Silene regia* forms a clade with the small, white-flowered *S. nivea* and *S. stellata* (Figure 3), and *S. virginica* again groups together with *S. polypetala* and *S. caroliniana.* The basal position of *S. californica* to all other North American taxa also indicates that Chowdhuri's (1957) section *Occidentales* is polyphyletic. The placement of *S. regia* differs in the ITS and *trn*L intron phylogenies. In the ITS phylogeny *S. regia* is in a strongly supported clade with *S. subciliata,* but in the *trn*L intron phylogeny it is in a weakly supported clade with *S. nivea,* which is lacking in the ITS phylogeny, and *S. stellata* (Figures 1 and 2). Major discrepancies between nuclear and chloroplast phylogenies may be evidence of hybridization in the recent history of the taxa (Avise 1994). Though the difference in the placement of *S. regia* does not conclusively demonstrate the importance of hybridization in the species history of the eastern North American *Silene,* it does deserve more investigation. The polyphyly of *S. caroliniana* in the ITS phylogeny may also indicate gene flow with closely related taxa. A more variable chloroplast marker and greater population sampling is needed to further examine reticulate evolution and resolve the relationships within the eastern North American *Silene.*

ACKNOWLEDGMENTS. This research was supported by a grant from the University of Missouri Conservation Biology Program to J.G.B. We are grateful to Janis Antonovics, Laura Galloway, Mary Puterbaugh, Robin Kennedy, and the Green Plant Market Nursery for their assistance in the tissue collection. Herbaria at the University of Missouri, Missouri Botanic Garden, and Northeast Louisiana University loaned specimens. We also thank Dean Bergstrom for helping with the sequencing and Sarah Mathews, Kim McBreen, and two anonymous reviewers for comments on the manuscript.

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